International Journal of Food Microbiology 157 (2012) 45–51

Contents lists available at SciVerse ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Pretreatment of the yeast antagonist, *Candida oleophila*, with glycine betaine increases oxidative stress tolerance in the microenvironment of apple wounds

Yuan Sui [a,1], Jia Liu [a,1], Michael Wisniewski [a,*,1], Samir Droby [b], John Norelli [a], Vera Hershkovitz [b]

[a] U.S. Department of Agriculture – Agricultural Research Service (USDA-ARS), Kearneysville, WV 25430, USA
[b] Agricultural Research Organization (ARO), The Volcani Center, Bet Dagan 50250, Israel

ARTICLE INFO

Article history:
Received 9 February 2012
Received in revised form 10 April 2012
Accepted 15 April 2012
Available online 20 April 2012

Keywords:
Antioxidant genes
Biocontrol yeast
Glycine betaine
Reactive oxygen species

ABSTRACT

In response to wounding, harvested fruit tissues of apple and citrus exhibit the production of reactive oxygen species (ROS). ROS production is greater when yeast antagonists used as biocontrol agents are applied in the wounds. These phenomena result in an oxidative stress environment for the yeast antagonists. It has been demonstrated that pre-exposure of some of these yeast antagonists to sublethal abiotic stress (heat or hydrogen peroxide), or stress-ameliorating compounds such as glycine betaine (GB) can induce subsequent oxidative stress tolerance in the antagonistic yeast. The increased level of oxidative stress tolerance has been demonstrated *in vitro* and is characterized by higher levels of antioxidant gene expression, increased production of trehalose, and lower levels of ROS when yeast are exposed to a subsequent oxidative stress. The current study determined whether or not the effects of GB on yeast antagonists determined *in vitro* persist and are present *in planta* when yeast are applied to wounded apples. The effect of exogenous GB on the production of ROS in the yeast antagonist, *Candida oleophila*, was determined after the yeast was placed in apple wounds. Oxidative damage to yeast cells recovered from apple wounds was also monitored. Results indicated that GB treatment improved the adaptation of *C. oleophila* to apple fruit wounds. Compared to untreated control yeast cells, GB-treated cells recovered from the oxidative stress environment of apple wounds exhibited less accumulation of ROS and lower levels of oxidative damage to cellular proteins and lipids. Additionally, GB-treated yeast exhibited greater biocontrol activity against *Penicillium expansum* and *Botrytis cinerea*, and faster growth in wounds of apple fruits compared to untreated yeast. The expression of major antioxidant genes, including *peroxisomal catalase*, *peroxiredoxin TSA1*, and *glutathione peroxidase* was elevated in the yeast by GB treatment. This study supports the premise that activation of antioxidant response in biocontrol yeast can improve biocontrol efficacy.
Published by Elsevier B.V.

1. Introduction

The use of biocontrol agents to manage postharvest diseases of fruits has shown great potential in recent years (Droby et al., 2011; Jamalizadeh et al., 2011; Sharma et al., 2009; Spadaro and Gullino, 2004). Among these agents, the utilization of yeasts has been emphasized (Janisiewicz et al., 2010; Wisniewski et al., 2007). The yeast antagonist, *Candida oleophila,* has demonstrated biocontrol efficacy against postharvest diseases of various fruits, including apple (Mercier and Wilson, 1995), peach (Droby et al., 2003), pear (Lahlali et al., 2011), grapefruit (Droby et al., 2002) and strawberry (Lima et al., 1997).

The ability to survive and proliferate in host tissues is crucial for the efficacy of antagonistic yeasts (Droby et al., 2009; Janisiewicz and Korsten, 2002). When applied to fruit surfaces and wounds, biocontrol agents encounter an oxidative stress environment that may affect their viability and efficacy (Castoria et al., 2003; Macarisin et al., 2010). The production of reactive oxygen species (ROS) in fruit tissue could in turn induce ROS accumulation in yeast cells, and result in oxidative damage thus compromising cell function (Garre et al., 2010; Saharan and Sharma, 2010). Macarisin et al. (2010) reported that *C. oleophila* and another biocontrol yeast, *Metschnikowia fructicola*, generated high levels of ROS when applied to intact or wounded apple fruits. Hershkovitz et al. (2012) reported that high amounts of ROS were detected in *M. fructicola* cells recovered from wounded grapefruit tissue. Therefore, enhancing tolerance to oxidative stress in fruit tissue may represent a useful strategy for improving the effectiveness of antagonistic yeasts.

As a major compatible solute, glycine betaine (GB, *N, N, N*-trimethyl glycine) plays a vital role in osmotic adjustment in various organisms, including bacteria, fungi, and plants (de Zwart et al., 2003; Rhodes and Hanson, 1993). GB has also been reported to have a protective function by inducing antioxidant compounds under stress conditions. For example, exogenous application of GB enhanced antioxidant scavenging compounds in tobacco (Banu et al., 2009), wheat (Raza et al.,

* Corresponding author at: U.S. Department of Agriculture – Agricultural Research Service (USDA-ARS), 2217 Wiltshire Road, Kearneysville, WV 25430, USA. Tel.: +1 304 725 3451; fax: +1 304 728 2340.
E-mail address: michael.wisniewski@ars.usda.gov (M. Wisniewski).
[1] These authors contributed equally to this work.

0168-1605/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.ijfoodmicro.2012.04.010

2007), rice (Farooq et al., 2008) and maize (Nawaz and Ashraf, 2010). In microorganisms, GB application has been shown to be an effective osmoprotectant in *Escherichia coli* (Cayley and Record, 2003), *Pichia pastoris* (Wang et al., 2007) and *Penicillium fellutanum* (Park and Gander, 1998). More recently, accumulation of GB and ectoine in osmotic stress adaptation of biocontrol bateria, *Bacillus amyloliquefaciens* and *Microbacterium oleovorans*, increased the potential for biocontrol of *Fusarium verticillioides* in maize (Sartori et al., 2012). GB was shown to improve H_2O_2-induced oxidative stress tolerance by elicitation of antioxidant enzyme activity in the biocontrol yeast, *Cystofilobasidium infirmominiatum* (Liu et al., 2011b). However, there is little information about the effect of GB on the antioxidant response of biocontrol yeast in the actual microenvironment of fruit wounds. The objective of the present study was to determine the effect of GB on the antioxidant response and biocontrol efficacy of *C. oleophila* after they were applied to apple wounds. More specifically, this study investigated the effect of a pretreatment of *C. oleophila* with GB on (i) subsequent ROS accumulation, protein carbonylation and lipid peroxidation in yeast recovered from apple wounds (ii) the induction of antioxidant genes, including *peroxisomal catalase* (*CAT*), *peroxiredoxin TSA1* (*TSA1*) and *glutathione peroxidase* (*GPX*) in *C. oleophila*, again in yeast recovered from apple wounds, and (iii) yeast viability in apple fruit wounds and biocontrol efficacy against *Penicillium expansum* and *Botrytis cinerea*.

2. Materials and methods

2.1. Yeast

C. oleophila (strain I-182) was isolated from the surface of tomato fruit as documented in a previous study (Wilson et al., 1993), and grown in a yeast peptone dextrose (YPD) broth (10 g of yeast extract, 20 g of peptone and 20 g of dextrose in 1000 ml of water). Twenty milliliters of YPD (Fisher Scientific, Fair Lawn, NJ, USA) was placed in 50-ml conical flasks and inoculated with *C. oleophila* at an initial concentration of 10^5 cells/ml determined with an automated cell counter, Cellometer Vision (Nexcelom Bioscience, Lawrence, MA, USA). Yeast cultures were incubated at 25 °C at 200 rpm on a rotary shaker (New Brunswick Scientific, Edison, NJ, USA).

2.2. Pathogens

P. expansum and *B. cinerea* were isolated from infected apple fruits and maintained on potato dextrose agar (PDA) (Difco, Sparks, MD, USA) at 4 °C (Liu et al., 2012). In order to reactivate the culture and verify their ability to cause disease, the pathogens were inoculated into wounds of apple fruit and re-isolated onto PDA once the infection was established. Spore suspensions of the two pathogens were obtained from 2-week-old cultures on PDA at 25 °C. The spore number was calculated with a Cellometer Vision, and the concentration was adjusted to 1×10^5 spores/ml with sterile distilled water.

2.3. Fruits

Apples (*Malus* x *domestica* Borkh. cv. Granny Smith) were harvested at commercial maturity. Fruits without wounds or rot were selected based on uniformity of size, disinfected with 2% (v/v) sodium hypochlorite for 2 min, rinsed with tap water and air dried (Li et al., 2008). These fruits were used in a subsequent biocontrol assay.

2.4. GB treatment of C. oleophila

Overnight yeast cultures were pelleted at 8000 *g* for 3 min and washed three times with sterile distilled water in order to remove residual medium, according to Li and Tian (2006). Washed cells were resuspended in the same volume (20 ml) of fresh YPD, supplemented with GB at the final concentration of 1 mM and incubated at 25 °C for 2 h at 200 rpm. The chosen concentration was based on previous studies (Boncompagni et al., 1999; Liu et al., 2011b) and a preliminary experiment. Control cells were subjected to the same process but without GB treatment. Cells were harvested by centrifugation at 8000 *g* for 3 min and washed three times with sterile distilled water in order to remove any residual GB and medium. The GB-treated (GB) and non-GB-treated (NGB) yeast samples were suspended in water at 5×10^7 cells/ml for the following experiments.

2.5. Determination of intracellular ROS accumulation in C. oleophila in response to apple fruit tissue

Three wounds (4 mm deep × 3 mm wide) were made on the equator of each fruit with a sterile nail. A 10 µl suspension of GB or NGB *C. oleophila* cells (5×10^7 cells/ml) was applied to each wound and allowed to air dry. Treated fruits were placed in a covered plastic food tray, and each tray was enclosed in a polyethylene bag and stored at 25 °C. Wound sites were removed using a 9 mm cork borer under aseptic condition after 6, 12, 24 and 48 h. The time point before inoculation into wounds served as time 0. Yeast cells were washed from 20 cylinders (4 mm deep) obtained from wounds of 10 fruits and cut into quarters, with 20 ml phosphate buffered saline (PBS, pH 7.0) in 50-ml conical flasks shaken at 200 rpm for 30 min on rotary shaker (Hershkovitz et al., 2012). Then yeast cells were pelleted at 8,000 g for 3 min and resuspended at the concentration of 1×10^7 cells/ml in 500 µl PBS containing 25 µM carboxy-H2DCFDA (Invitrogen, Eugene, OR, USA), and the suspensions were incubated in the dark at 30 °C for 1 h (Liu et al., 2011a, b). The yeast cells were then washed twice with PBS buffer and resuspended in 96-well plate (200 µl PBS per well). The fluorescence intensity was measured at an excitation of 485 nm and emission of 530 nm using a fluorescence microplate reader (Synergy™ HT, BioTek Instruments, Winooski, VT, USA). The values of NGB and GB cells at different time points were calculated as the relative fluorescence intensity, compared to NGB cells at time 0 (Dirmeier et al., 2002; Oh and Lim, 2008). There were three replicates in each treatment, and the experiment was repeated three times.

2.6. Determination of protein carbonylation and lipid peroxidation in C. oleophila

Yeast samples (GB or NGB) were collected from fruit wounds at 0, 6, 12, 24 and 48 h using the same method as described above. Yeast cells were disrupted in liquid nitrogen with the aid of a mortar and pestle. Protein carbonylation was measured as an indicator of oxidative damage to proteins (Liu et al., 2011b). Proteins were extracted from the samples using 500 µl of 50 mM KH_2PO4 buffer (pH 7.5) containing 10 mM Tris, 2 mM $MgCl_2$, 2 mM EGTA and 1 mM PMSF. Aliquots of extract were reacted with 500 µl of 10 mM 2,4-dinitrophenylhydrazine (DNPH) dissolved in 2.5 M HCl or 2.5 M HCl without DNPH (blank control) in the dark at room temperature with vortex every 15 min for 1 h. Proteins were precipitated with 20% trichloroacetic acid (TCA, w/v) and kept on ice for 10 min. After centrifuging at 3000 *g* for 20 min, protein pellets were washed with ethanol-ethyl acetate (1:1, v/v) and dissolved in 6 M guanidine hydrochloride with 20 mM KH_2PO_4 (pH 2.3). The absorbance was recorded at 380 nm after centrifugation at 9500 *g* for 10 min. The carbonyl content was calculated using the molar absorption coefficient of 22,000/M/cm, and expressed as nmol per mg protein.

To assay lipid peroxidation, a method based on the reaction of thiobarbituric acid with malondialdehyde (MDA) was used. Detection of thiobarbituric acid-reactive species (TBARS) was carried out as described by Garre et al. (2010). Disrupted yeast samples were resuspended in 500 µl of 50 mM KH_2PO4 buffer (pH 6.0) containing 10% (w/v) trichloroacetic acid, and centrifuged at 3,000 g for 10 min.

Supernatants were mixed with 100 μl of 0.1 M EDTA and 600 μl of 1% (w/v) thiobarbituric acid. After cooling down, the absorbance was measured at 532 nm. The MDA content was calculated using the molar absorption coefficient of 153,000/M/cm, and expressed as nmol per mg protein.

Protein content was measured using the Bradford assay (Bradford, 1976). Bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) was used as a standard. There were three replicates in each treatment, and the experiment was repeated three times.

2.7. RNA isolation and semi-quantitative RT-PCR analysis of antioxidant gene expression

Total RNA from yeast samples (NGB or GB) collected from fruit wounds at 0, 6, 12, 24 and 48 h was isolated using an RNeasy Mini Kit (Qiagen Science, Germantown, MD, USA) according to the manufacturer's instructions (Liu et al., 2011a). Extracted RNA was treated with TURBO™ DNase (Ambion, Austin, TX, USA) and purified again with RNeasy. Aliquots of 1 μg total RNA were used for first strand cDNA synthesis in 20 μl reaction volume with 100 units of M-MLV reverse transcriptase (Ambion, Austin, TX, USA). Transcript levels of 18S rRNA gene served as an internal control (Lee et al., 2008; Pacheco et al., 2009). Cycling parameters for each gene amplification were 95 °C for 5 min; 25 cycles of 95 °C for 30 s, specific annealing temperature for 30 s, and 72 °C for 30 s; and finally 72 °C for 10 min. The primer pairs and annealing temperatures of the antioxidant genes *CAT*, *TSA1* and *GPX* were designed according to Liu et al. (2012). PCR products were cloned and sequenced to verify the identity. Quantification of transcript expression level was based on the band intensity on an ethidium-bromide-stained gel using Scion Image Software (Scion Corp., Frederick, Maryland, USA) according to Oliveira et al. (2010). There were three replicates in each treatment, and the experiment was repeated three times.

2.8. Population dynamics of C. oleophila in wounds of apple fruits

Three wounds (4 mm deep × 3 mm wide) were made on the equator of each fruit with a sterile nail. A 10 μl suspension of GB or NGB cells of *C. oleophila* (5×10^7 cells/ml) was applied to each wound. Fruit samples were then collected at 6, 12, 24 and 48 h after treatment and yeast populations were determined as described by Cao et al. (2010). Briefly, yeasts were recovered by removing 20 samples of wounded tissues of 10 fruits with a 9 mm cork borer. Samples were then ground with a mortar and pestle in 20 ml sterile distilled water. Then, 50 μl of serial 10-fold dilutions were spread on YPD agar plates. Samples taken at 1 h after treatment served as time 0. Colonies were counted after incubation at 25 °C for 3 days and expressed as the Log_{10} CFU per wound. There were three replicates in each treatment, and the experiment was repeated three times.

2.9. Biocontrol assay of C. oleophila against postharvest diseases of apple fruits

Biocontrol efficacy was tested according to a previous study (Liu et al., 2009). Three wounds (4 mm deep × 3 mm wide) were made on the equator of each fruit with a sterile nail. A 10 μl suspension of non-GB-treated (NGB) or GB-treated (GB) cells of *C. oleophila* (5×10^7 cells/ml) was applied to each wound. Sterile distilled water served as a control. When fruits were air-dried, 10 μl of either *P. expansum* or *B. cinerea* suspension (1×10^5 spores/ml) were inoculated into each wound. Treated fruits were placed in a covered plastic food tray. Each tray was enclosed with a polyethylene bag in order to maintain high humidity (about 95% relative humidity) and stored at 25 °C. Disease incidence and lesion diameter of apple fruits were recorded after 4 days. Each treatment contained three replicates with ten fruits per replicate and the experiment was repeated three times.

2.10. Data analysis

All statistical analyses were performed with SPSS version 13.0 (SPSS Inc., Chicago, IL, USA). Two-way analysis of variance (ANOVA) analysis was performed on data where treatment and time were variables, and mean separation was determined for non-GB-treated control (NGB) and GB-treated (GB) yeast samples at each time point using a Student's *t*-test. Data with a single variable (treatment) in biocontrol test were analyzed by one-way ANOVA, and mean separations were performed by Duncan's multiple range tests. Differences at $P<0.05$ were considered significant. Data presented in this paper were pooled across three independent repeated experiments, as the interaction between treatment and experiment variables was not significant.

3. Results

3.1. ROS accumulation of C. oleophila

At time 0, prior to inoculation into apple wounds, ROS levels in both GB and NGB cells were at a low level (Fig. 1). ROS accumulation peaked in yeast cells recovered from apple wounds at 6 h after inoculation and gradually decreased over the 48 h period in which samples were taken. Even at 48 h, ROS levels were still above those observed at 0 h. Both treatment and duration of time in the wounds had a significant ($P<0.05$, two-way ANOVA analysis) effect on intracellular ROS accumulation in *C. oleophila*. GB-treated cells exhibited a significantly ($P<0.05$, Student's *t*-test) lower ROS level compared to NGB cells at all of the four time points (after time 0) in fruit wounds. After 48 h at fruit wounds, the relative value of ROS level in GB cells was 1.43 while that of NGB cells was 1.84.

3.2. Protein carbonylation and lipid peroxidation

To investigate the protective effect of GB against oxidative damage to proteins and lipids, carbonyl content (indicator of protein oxidation) and MDA content (indicator of lipid peroxidation) in *C. oleophila* cells recovered from wounds were measured (Fig. 2). The pattern in the levels of both carbonyl and MDA content were similar to what was observed for ROS accumulation. Levels of MDA were higher than carbonyl content, although both parameters increased by approximately three-

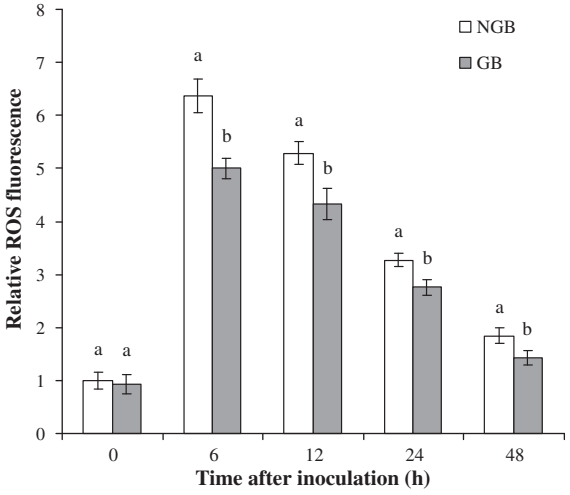

Fig. 1. ROS accumulation in non-glycine betaine-treated (NGB) and glycine betaine-treated (GB) cells of *C. oleophila* recovered from apple wounds at varying periods of time following inoculation. The relative ROS fluorescence values of NGB and GB cells at different time points were calculated as the relative fluorescence intensity compared to NGB cells at time 0. Data presented are the means ± sd of three independent experiments where each experiment consisted of three biological replicates for a total of n = 9. Columns with different letters at each time point indicate significant differences according to Student's *t*-test ($P<0.05$).

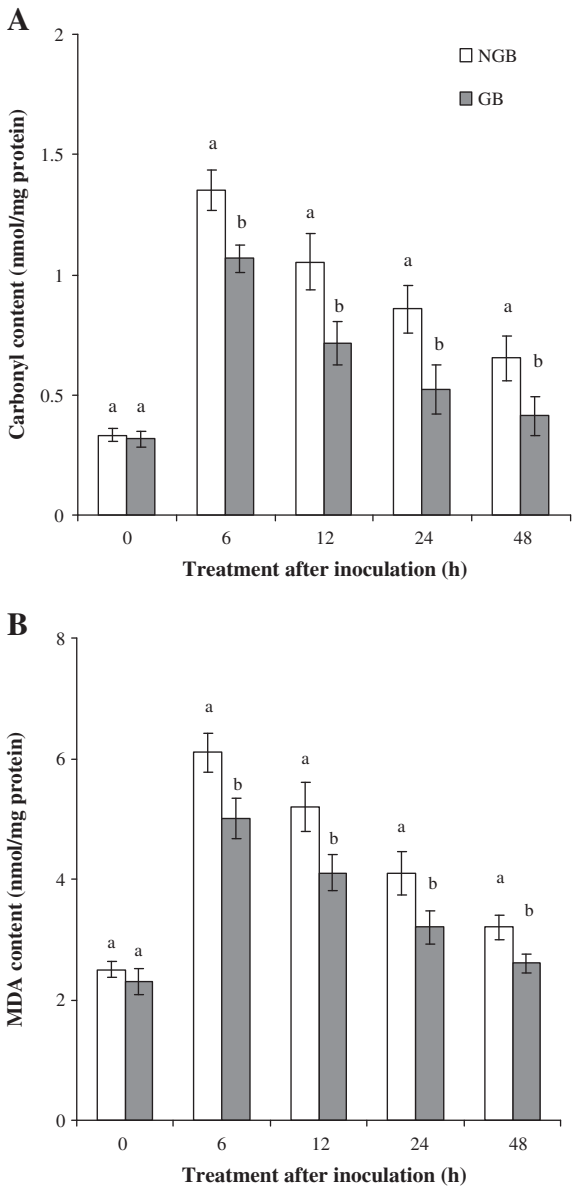

Fig. 2. Protein carbonylation (A) and lipid peroxidation (B) in non-glycine betaine-treated (NGB) and glycine betaine-treated (GB) cells of *C. oleophila* recovered from apple wounds at varying periods of time following inoculation. Data presented are the means ± sd of nine pooled replicates. Columns with different letters at each time point indicate significant differences according to Student's *t*-test ($P<0.05$).

fold at their maximum. At time 0, prior to inoculation into apple wounds, carbonyl and MDA contents in NGB and GB cells were low and not significantly different in GB and NGB cells. Inoculation of yeast into apple wounds resulted in a marked increase in both carbonyl and MDA content during the first 12 hours. While elevated in both, GB cells had a significantly ($P<0.05$, Student's *t*-test) lower carbonyl content and MDA content than NGB cells at all of the four time points after time 0.

3.3. Antioxidant gene expression

Sequencing results confirmed that the PCR product obtained from each pair of primers was 100% identical to each target gene (data not shown). Semi-quantitative analysis of gene expression of yeast samples at time 0 (the time point after 2-h GB treatment but prior to inoculation into fruit wounds) indicated that GB treatment induced the expression of *CAT*, *TSA1* and *GPX* in *C. oleophila* cells (Fig. 3). The inductive effect was

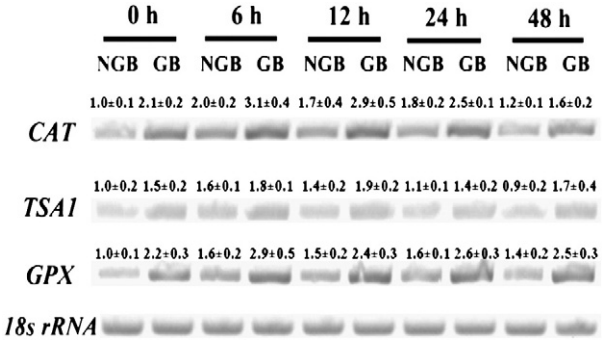

Fig. 3. Expression of some antioxidant genes (*CAT*, *TSA1* and *GPX*) in non-glycine betaine-treated (NGB) and glycine betaine-treated (GB) cells of *C. oleophila* recovered from apple wounds at varying periods of time following inoculation. The 18S rRNA gene was used as a control for normalizing mRNA quantity. The mean level of gene expression, as determined by densitometry, relative to the sample at Time 0 is shown above each band ($n=9\pm$sd). GB cells had significantly higher levels of expression compared to NGB cells for all three genes at all the time periods evaluated ($P<0.05$, Student's *t*-test).

more apparent for *CAT* and *GPX* than with *TSA1*. Moreover, the inductive effect of GB treatment on *C. oleophila* was evident at least 2 d after inoculation into apple wounds (Fig. 3). Statistical analysis indicated that GB yeast cells had higher levels of expression compared to NGB cells for all three genes at all time periods measured ($P<0.05$, Student's *t*-test).

3.4. Population dynamics of *C. oleophila* in apple wounds

Regardless of whether or not the yeast cells had been pre-treated with GB, *C. oleophila* multiplied quickly in apple fruit wounds (Fig. 4). The GB treatment, however, did have a significant impact on yeast growth in apple wounds ($P<0.05$, two-way ANOVA analysis). After 12 h, the number of the GB and NGB yeast increased nearly ten-fold. The population of GB-treated cells, however, was significantly ($P<0.05$, Student's *t*-test) higher than the population of NGB-treated cells at all the time points after time 0.

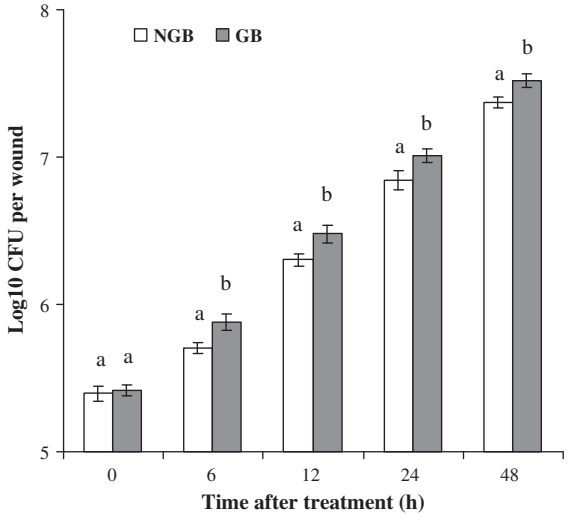

Fig. 4. Population dynamics of *C. oleophila* in wounds of apple fruits stored at 25 °C. Fruits were wounded and inoculated with 10 μl of non-glycine betaine-treated (NGB) or glycine betaine-treated (GB) cell suspension of *C. oleophila* at 5×10^7 cells/ml. Yeast were recovered from wounds at varying periods of time following inoculation and colonies were counted after incubation at 25 °C for 3 days and expressed as the Log_{10} CFU per wound. Data presented are the means ± sd of nine pooled replicates. Columns with different letters at each time point indicate significant differences according to Student's *t*-test ($P<0.05$).

3.5. Biocontrol efficacy of C. oleophila

As shown in Fig. 5, *C. oleophila* significantly reduced both disease incidence and lesion diameter of both blue mold and grey mold of apple caused by *P. expansum* and *B. cinerea*, respectively. Importantly, GB yeast exhibited a greater level of efficacy than NGB yeast for both disease incidence and lesion diameter ($P<0.05$, one-way ANOVA).

4. Discussion

The oxidative environment plays an important role in biocontrol systems involving a biocontrol agent, pathogen, and host (Hershkovitz et al., 2012; Macarisin et al., 2007, 2010). Cells of fruit tissue bordering the edge of wounds undergo an oxidative burst producing high levels of hydrogen peroxide (Macarisin et al., 2007) while yeast antagonists placed on the surface or within wounds of fruit also exhibit a large increase in ROS (Macarisin et al., 2010). The application of yeast to wounded fruit tissue will also stimulate host ROS production over and above the level induced by wounding alone (Macarisin et al., 2010). Thus there is considerable interaction between the biocontrol agent and host in determining the redox environment at the very site where the biocontrol agent is expected to inhibit and control a specific pathogen. Additionally, the redox environment can either stimulate or be detrimental to different fungal pathogens (Lee and Bostock, 2007; Williams et al., 2011). Regardless of the source of ROS production, it appears that yeast antagonists used to control postharvest diseases of fruits are exposed to oxidative stress (Macarisin et al., 2010; Tolaini et al., 2010). Because of this, Castoria et al. (2003) indicated that microbial antagonists with a high level of oxidative stress tolerance have greater potential as postharvest biocontrol agents. Alternatively, improving the oxidative stress tolerance of biocontrol agents during the formulation process (Liu et al., 2009) or pretreatment of the antagonist prior to application with either a sublethal stress or stress-ameliorating compound (Liu et al., 2011a & b) may also represent a useful strategy for increasing the performance of postharvest biocontrol yeasts.

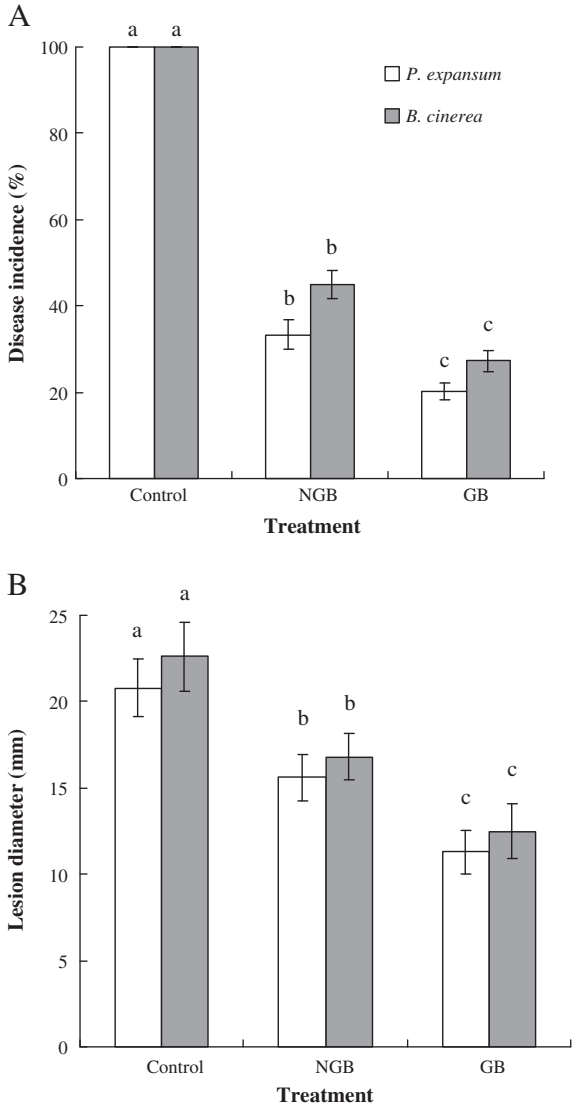

Fig. 5. Biocontrol efficacy of *C. oleophila* against *P. expansum* and *B. cinerea* on apple fruits stored at 25 °C. Fruits were wounded and inoculated with 10 µl of sterile water (control), or either a non-glycine betaine-treated (NGB) or glycine betaine-treated (GB) cell suspension of *C. oleophila* at 5×10^7 cells/ml. Fruits were air-dried and then wounds were inoculated with either 10 µl of *P. expansum* or *B. cinerea* at 1×10^5 spores/ml. Disease incidence (A) and lesion diameter (B) in apple fruits were recorded after 4 days. Data presented are the means ± sd of nine pooled replicates. Columns with different letters indicate significant differences according to Duncan's multiple range test ($P<0.05$).

The main objective of the present study was to determine if the oxidative stress tolerance induced in yeast *in vitro* by pretreatment with a stress-ameliorating compound (glycine betaine) would persist in yeast after they were applied and subsequently recovered from wounded fruit tissues. In a previous *in vitro* study, utilizing the antagonist *C. infirmominiatum*, glycine betaine was shown to be effective in reducing ROS accumulation (Liu et al., 2011b). In the current study, the beneficial effect of glycine betaine in improving oxidative stress tolerance was demonstrated in *C. oleophila* after they were inoculated and recovered from apple wounds (Fig. 1). The results from both studies support the premise that oxidative stress tolerance in antagonistic yeast can be increased by exposure to exogenous GB and that the increased level of tolerance is associated with the enhancement of biocontrol efficacy. Exogenous GB has been reported to modulate antioxidant enzymes and improve stress tolerance in plants (Farooq et al., 2008; Nawaz and Ashraf, 2010; Raza et al., 2007), animal cells (Alfieri et al., 2002; Monobe et al., 2006), and human cells (Craig, 2004). Additionally, intracellular accumulation of GB was shown to be associated with improved osmotic and thermal stress tolerance in the bacterium, *Pantoea agglomerans*, which is used as a postharvest biocontrol agent (Cañamás et al., 2007; Teixidó et al., 2005).

High levels of ROS, in the absence of an effective scavenging system, can cause oxidative damage to cell components including proteins, lipids and nucleic acids, resulting in compromised cell function (Branduardi et al., 2007; Reverter-Branchat et al., 2004). The level of carbonyl groups present and MDA content are widely used as indicators of oxidative damage to proteins and lipids, respectively (Abegg et al., 2010; Li et al., 2010). The data in Fig. 2 indicated that lower carbonyl and MDA levels were detected in GB cells compared to NGB cells recovered from apple wounds. These findings are consistent with our previous finding that GB treatment significantly reduced protein carbonylation and lipid peroxidation in *C. infirmominiatum in vitro* when the yeast was exposed to oxidative stress (Liu et al., 2011b). Banu et al. (2009) also found that GB treatment reduced ROS accumulation and lipid peroxidation in tobacco cells under salt stress. The reduced oxidative damage to yeast cells may be due to the function of glycine betaine reported by Einset and Connolly (2009). They suggested that GB blocks ROS signaling by activating antioxidant genes thus reducing the accumulation of ROS.

The enzymatic detoxification of ROS is dependent on the upregulation of antioxidant genes such as *CAT*, *TSA1* and *GPX* (Collinson et al., 2002; Nakagawa et al., 2010; Wu et al., 2007). Our data indicate that GB treatment upregulated the expression of these three genes in *C. oleophila* cells and that elevated expression of these genes persisted after the yeast were inoculated into apple wounds (Fig. 3). Similar results have been reported by Banu et al. (2009), who reported that GB enhanced the gene expression of *CAT* and lignin-forming peroxidase in tobacco cells under salt stress. Therefore, the induction of antioxidant

gene expression in *C. oleophila* by exogenous GB may be a key factor in lowering ROS levels and ameliorating oxidative damage, thus improving the viability of yeast cells in fruit wounds.

Cell viability has a direct influence on the population dynamics of biocontrol agents in fruit wounds and high population density is considered an advantage in competing for nutrients and space, both of which play a major role in biocontrol efficacy (Droby et al., 2009; Wisniewski et al., 2007). It is notable that the population of GB-treated cells of *C. oleophila* grew more rapidly than NGB-treated cells in wounds of apple fruits during the 48 h time period sampled (Fig. 4). This observation is in agreement our previous report on the stimulating effect of GB on growth of another yeast species, *C. infirmominiatum* (Liu et al., 2011b), as well as the reported effect on bacteria (Boniolo et al., 2009; Delamare et al., 2003). The first 48 hours after inoculation in apple wounds is crucial for pathogen germination and infection (Li et al., 2008). Thus, the higher population of GB-treated yeast cells in this time period, compared to NGB-treated cells (Fig. 5), may also have contributed to the improved performance in biocontrol of both *B. cinerea* and *P. expansum*.

In conclusion, we found that a pretreatment of the yeast, *C. oleophila*, with glycine betaine could reduce intracellular ROS accumulation, as well as protein and lipid oxidation even after the yeast were applied and then recovered from apple wounds. The increased level of oxidative stress tolerance persisted for at least 48 h following wound inoculation. The reduction in ROS accumulation and oxidative damage resulting from the GB treatment was associated with the upregulation of several antioxidant genes.

Acknowledgements

This research was partially supported by a grant (IS-4268-09) from the U.S.–Israel Binational Agricultural Research and Development (BARD) Fund to S. D. and M. W.

References

Abegg, M.A., Alabarse, P.V.G., Casanova, A., Hoscheid, J., Salomon, T.B., Hackenhaar, F.S., Medeiros, T.M., Benfato, M.S., 2010. Response to oxidative stress in eight pathogenic yeast species of the genus *Candida*. Mycopathologia 170, 11–20.

Alfieri, R.R., Cavazzoni, A., Petronini, P.G., Bonelli, M.A., Caccamo, A.E., Borghetti, A.F., Wheeler, K.P., 2002. Compatible osmolytes modulate the response of porcine endothelial cells to hypertonicity and protect them from apoptosis. The Journal of Physiology 540, 499–508.

Banu, N.A., Hoque, A., Watanabe-Sugimoto, M., Matsuoka, K., Nakamura, Y., Shimoishi, Y., Murata, Y., 2009. Proline and glycinebetaine induce antioxidant defense gene expression and suppress cell death in cultured tobacco cells under salt stress. Journal of Plant Physiology 166, 146–156.

Boncompagni, E., Østerås, M., Poggi, M.C., Rudulier, D.L., 1999. Occurrence of choline and glycine betaine uptake and metabolism in the family *Rhizobiaceae* and their roles in osmoprotection. Applied and Environmental Microbiology 65, 2072–2077.

Boniolo, F.S., Rodrigues, R.C., Delatorre, E.O., da Silveira, M.M., Flores, V.M., Berbert-Molina, M.A., 2009. Glycine betaine enhances growth of nitrogen-fixing bacteria *Gluconacetobacter diazotrophicus* PAL5 under saline stress conditions. Current Microbiology 59, 593–595.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72, 248–254.

Branduardi, P., Fossati, T., Sauer, M., Pagani, R., Mattanovich, D., Porro, D., 2007. Biosynthesis of vitamin C by yeast leads to increased stress resistance. PLoS One 2, e1092.

Cañamás, T.P., Viñas, I., Usall, J., Magan, N., Morelló, J.R., Teixidó, N., 2007. Relative importance of amino acids, glycine-betaine and ectoine synthesis in the biocontrol agent *Pantoea agglomerans* CPA-2 in response to osmotic, acidic and heat stress. Letters in Applied Microbiology 45, 6–12.

Cao, S., Yuan, Y., Hu, Z., Zheng, Y., 2010. Combination of *Pichia membranifaciens* and ammonium molybdate for controlling blue mould caused by *Penicillium expansum* in peach fruit. International Journal of Food Microbiology 141, 173–176.

Castoria, R., Caputo, L., De Curtis, F., De Cicco, V., 2003. Resistance of postharvest biocontrol yeasts to oxidative stress: a possible new mechanism of action. Phytopathology 93, 564–572.

Cayley, S., Record, M.T., 2003. Roles of cytoplasmic osmolytes, water, and crowding in response of *Escherichia coli* to osmotic stress: biophysical basis of osmoprotection by glycine betaine. Biochemistry 42, 12596–12609.

Collinson, E.J., Wheeler, G.L., Garrido, E.O., Avery, A.M., Avery, S.V., Grant, C.M., 2002. The yeast glutaredoxins are active as glutathione peroxidases. Journal of Biological Chemistry 277, 16712–16717.

Craig, S.A.S., 2004. Betaine in human nutrition. American Journal of Clinical Nutrition 80, 539–549.

de Zwart, F.J., Slow, S., Payne, R.J., Lever, M., George, P.M., Gerrard, J.A., Chambers, S.T., 2003. Glycine betaine and glycine betaine analogues in common foods. Food Chemistry 83, 197–204.

Delamare, A.P.L., Dalcin, T., Müller, G., Olavo, S., da Costa, P., Echeverrigaray, S., 2003. The effect of organic osmoprotectors on *Aeromonas trota* and *A. hydrophila* grown under high sodium chloride concentrations. Brazilian Journal of Microbiology 34, 128–130.

Dirmeier, R., O'Brien, K.M., Engle, M., Dodd, A., Spears, E., Poyton, R.O., 2002. Exposure of yeast cells to anoxia induces transient oxidative stress. Journal of Biological Chemistry 277, 34773–34784.

Droby, S., Vinokur, V., Weiss, B., Cohen, L., Daus, A., Goldschmidt, E.E., Porat, R., 2002. Induction of resistance to *Penicillium digitatum* in grapefruit by the yeast biocontrol agent *Candida oleophila*. Phytopathology 92, 393–399.

Droby, S., Wisniewski, M., El Ghaouth, A., Wilson, C., 2003. Influence of food additives on the control of postharvest rots of apple and peach and efficacy of the yeast-based biocontrol product Aspire. Postharvest Biology and Technology 27, 127–135.

Droby, S., Wisniewski, M., Macarisin, D., Wilson, C., 2009. Twenty years of postharvest biocontrol research: Is it time for a new paradigm? Postharvest Biology and Technology 52, 137–145.

Droby, S., Wisniewski, M., Benkeblia, N., 2011. Postharvest pathology of tropical and subtropical fruit and strategies for decay control. In: Yahia, E.M. (Ed.), Postharvest biology and technology of tropical and subtropical fruits. : Fundamental issues, 1. Woodhead Publishing, Sawston, Cambridge, UK, pp. 194–216.

Einset, J., Connolly, E.L., 2009. Glycine betaine enhances extracellular processes blocking ROS signaling during stress. Plant Signaling & Behavior 4, 197–199.

Farooq, M., Basra, S.M.A., Wahid, A., Cheema, Z.A., Cheema, M.A., Khaliq, A., 2008. Physiological role of exogenously applied glycinebetaine to improve drought tolerance in fine grain aromatic rice (*Oryza sativa* L.). Journal of Agronomy and Crop Science 194, 325–333.

Garre, E., Raginel, F., Palacios, A., Julien, A., Matallana, E., 2010. Oxidative stress responses and lipid peroxidation damage are induced during dehydration in the production of dry active wine yeasts. International Journal of Food Microbiology 136, 295–303.

Hershkovitz, V., Ben-Dayan, C., Raphael, G., Pasmanik-Chor, M., Liu, J., Belausov, E., Aly, R., Wisniewski, M., Droby, S., 2012. Global changes in gene expression of grapefruit peel tissue in response to the yeast biocontrol agent *Metschnikowia fructicola*. Molecular Plant Pathology 13, 338–349.

Jamalizadeh, M., Etebarian, H.R., Aminian, H., Alizadeh, A., 2011. A review of mechanisms of action of biological control organisms against post-harvest fruit spoilage. EPPO Bulletin 41, 65–71.

Janisiewicz, W.J., Korsten, L., 2002. Biological control of postharvest diseases of fruits. Annual Review of Phytopathology 40, 411–441.

Janisiewicz, W.J., Kurtzman, C.P., Buyer, J.S., 2010. Yeasts associated with nectarines and their potential for biological control of brown rot. Yeast 27, 389–398.

Lahlali, R., Raffaele, B., Jijakli, M.H., 2011. UV protectants for *Candida oleophila* (strain O), a biocontrol agent of postharvest fruit diseases. Plant Pathology 60, 288–295.

Lee, M.H., Bostock, R.M., 2007. Fruit exocarp phenols in relation to quiescence and development of *Monilinia fructicola* infections in *Prunus* spp.: a role for cellular redox? Phytopathology 97, 269–277.

Lee, P., Cho, B.R., Joo, H.S., Hahn, J.S., 2008. Yeast Yak1 kinase, a bridge between PKA and stress-responsive transcription factors, Hsf1 and Msn2/Msn4. Molecular Microbiology 70, 882–895.

Li, B.Q., Tian, S.P., 2006. Effects of trehalose on stress tolerance and biocontrol efficacy of *Cryptococcus laurentii*. Journal of Applied Microbiology 100, 854–861.

Li, B.Q., Zhou, Z.W., Tian, S.P., 2008. Combined effects of endo- and exogenous trehalose on stress tolerance and biocontrol efficacy of two antagonistic yeasts. Biological Control 46, 187–193.

Li, H., Wang, H.L., Du, J., Du, G., Zhan, J.C., Huang, W.D., 2010. Trehalose protects wine yeast against oxidation under thermal stress. World Journal of Microbiology and Biotechnology 26, 969–976.

Lima, G., Ippolito, A., Nigro, F., Salerno, M., 1997. Effectiveness of *Aureobasidium pullulans* and *Candida oleophila* against postharvest strawberry rots. Postharvest Biology and Technology 10, 169–178.

Liu, J., Tian, S., Li, B., Qin, G., 2009. Enhancing viability of two biocontrol yeasts in liquid formulation by applying sugar protectant combined with antioxidant. Biocontrol 54, 817–824.

Liu, J., Wisniewski, M., Droby, S., Tian, S., Hershkovitz, V., Tworkoski, T., 2011a. Effect of heat shock treatment on stress tolerance and biocontrol efficacy of *Metschnikowia fructicola*. FEMS Microbiology Ecology 76, 145–155.

Liu, J., Wisniewski, M., Droby, S., Vero, S., Tian, S., Hershkovitz, V., 2011b. Glycine betaine improves oxidative stress tolerance and biocontrol efficacy of the antagonistic yeast *Cystofilobasidium infirmominiatum*. International Journal of Food Microbiology 146, 76–83.

Liu, J., Wisniewski, M., Droby, S., Norelli, J., Hershkovitz, V., Tian, S., Farrell, R., 2012. Increase in antioxidant gene transcripts, stress tolerance and biocontrol efficacy of *Candida oleophila* following sublethal oxidative stress exposure. FEMS Microbiology Ecology doi:10.1111/j.1574–6941.2012.01324.x.

Macarisin, D., Cohen, L., Eick, A., Rafael, G., Belausov, E., Wisniewski, M., Droby, S., 2007. *Penicillium digitatum* suppresses production of hydrogen peroxide in host tissue during infection of citrus fruit. Phytopathology 97, 1491–1500.

Macarisin, D., Droby, S., Bauchan, G., Wisniewski, M., 2010. Superoxide anion and hydrogen peroxide in the yeast antagonist-fruit interaction: A new role for reactive oxygen species in postharvest biocontrol? Postharvest Biology and Technology 58, 194–202.

Mercier, J., Wilson, C.L., 1995. Effect of wound moisture on the biocontrol by *Candida oleophila* of gray mold rot (*Botrytis cinerea*) of apple. Postharvest Biology and Technology 6, 9–15.

Monobe, M., Hamano, N., Sumi, M., Mukai, K., Moritake, T., Anzai, K., Uzawa, A., Ando, K., 2006. Effects of glycine betaine on bone marrow death and intestinal damage by gamma rays and carbon ions. Radiation Protection Dosimetry 122, 494–497.

Nakagawa, T., Yoshida, K., Takeuchi, A., Ito, T., Fujimura, S., Matsufuji, Y., Tomizuka, N., Yurimoto, H., Sakai, Y., Hayakawa, T., 2010. The peroxisomal catalase gene in the methylotrophic yeast *Pichia methanolica*. Bioscience, Biotechnology, and Biochemistry 74, 1733–1735.

Nawaz, K., Ashraf, M., 2010. Exogenous application of glycinebetaine modulates activities of antioxidants in maize plants subjected to salt stress. Journal of Agronomy and Crop Science 196, 28–37.

Oh, P.S., Lim, K.T., 2008. Blocking of intracellular ROS production by phytoglycoprotein (30 kDa) causes anti-proliferation in bisphenol A-stimulated Chang liver cells. Journal of Applied Toxicology 28, 749–758.

Oliveira, M.B., Nascimento, L.B., Junior, M.L., Petrofeza, S., 2010. Characterization of the dry bean polygalacturonase-inhibiting protein (PGIP) gene family during *Sclerotinia sclerotiorum* (Sclerotiniaceae) infection. Genetics and Molecular Research 9, 994–1004.

Pacheco, A., Pereira, C., Almeida, M.J., Soura, M.J., 2009. Small heat-shock protein Hsp12 contributes to yeast tolerance to freezing stress. Microbiology 155, 2021–2028.

Park, Y.I., Gander, J.E., 1998. Choline derivatives involved in osmotolerance of *Penicillium fellutanum*. Applied and Environmental Microbiology 64, 273–278.

Raza, S.H., Athar, H.R., Ashraf, M., Hameed, A., 2007. Glycinebetaine-induced modulation of antioxidant enzymes activities and ion accumulation in two wheat cultivars differing in salt tolerance. Environmental and Experimental Botany 60, 368–376.

Reverter-Branchat, G., Cabiscol, E., Tamarit, J., Ros, J., 2004. Oxidative damage to specific proteins in replicative and chronological-aged *Saccharomyces cerevisiae*: common targets and prevention by calorie restriction. Journal of Biological Chemistry 279, 31983–31989.

Rhodes, D., Hanson, A.D., 1993. Quaternary ammonium and tertiary sulfonium compounds in higher plants. Annual Review of Plant Physiology and Plant Molecular Biology l44, 357–384.

Saharan, R.K., Sharma, S.C., 2010. Correlation studies of trehalose with oxidative stress in ethanol stressed yeast *Pachysolen tannophilus*. Current Research Journal of Biological Sciences 2, 300–305.

Sartori, M., Nesci, A., Magan, N., Etcheverry, M., 2012. Accumulation of the betaine and ectoine in osmotic stress of biocontrol agents against *Fusarium verticillioides* in maize. Agricultural Science 3, 83–89.

Sharma, R.R., Singh, D., Singh, R., 2009. Biological control of postharvest diseases of fruits and vegetables by microbial antagonists: A review. Biological Control 50, 205–221.

Spadaro, D., Gullino, M.L., 2004. State of the art and future prospects of the biological control of postharvest fruit diseases. International Journal of Food Microbiology 91, 185–194.

Teixidó, N., Cañamás, T.P., Usall, J., Torres, R., Magan, N., Viñas, I., 2005. Accumulation of the compatible solutes, glycine-betaine and ectoine, in osmotic stress adaptation and heat shock cross-protection in the biocontrol agent *Pantoea agglomerans* CPA-2. Letters in Applied Microbiology 41, 248–252.

Tolaini, V., Zjalic, S., Reverberi, M., Fanelli, C., Fabbri, A.A., Del Fiore, A., De Rossi, P., Ricelli, A., 2010. *Lentinula edodes* enhances the biocontrol activity of *Cryptococcus laurentii* against *Penicillium expansum* contamination and patulin production in apple fruits. International Journal of Food Microbiology 138, 243–249.

Wang, S., Yao, Q., Tao, J., Qiao, Y., Zhang, Z., 2007. Co-ordinate expression of glycine betaine synthesis genes linked by the FMDV 2A region in a single open reading frame in *Pichia pastoris*. Applied Microbiology and Biotechnology 77, 891–899.

Williams, B., Kabbage, M., Kim, H.J., Britt, R., Dickman, M.B., 2011. Tipping the balance: *Sclerotinia sclerotiorum* secreted oxalic acid suppresses host defenses by manipulating the host redox environment. PLoS Pathogens 7, e1002107.

Wilson, C., Wisniewski, M., Droby, S., Chalutz, E., 1993. A selection strategy for microbial antagonists to control postharvest diseases of fruits and vegetables. Scientia Horticulturae 53, 183–189.

Wisniewski, M., Wilson, C., Droby, S., Chalutz, E., El Ghaouth, A., Stevens, C., 2007. Postharvest biocontrol: new concepts and applications. In: Vincent, C., Goettal, M.S., Lazarovits, G. (Eds.), Biological Control: A Global Perspective. CABI, Cambridge, MA, USA, pp. 262–273.

Wu, C.Y., Bird, A.J., Winge, D.R., Eide, D.J., 2007. Regulation of the yeast TSA1 peroxiredoxin by ZAP1 is an adaptive response to the oxidative stress of zinc deficiency. Journal of Biological Chemistry 282, 2184–2198.